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Cell Cycle Regulated Transport Controlled by Alterations in the Nuclear Pore Complex

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Summary

Eukaryotic cells have developed mechanisms for regulating the nuclear transport of macromolecules that control various cellular events including movement through defined stages of the cell cycle. In yeast cells, where the nuclear envelope remains intact throughout the cell cycle, these transport regulatory mechanisms must also function during mitosis. We have uncovered a mechanism for regulating transport that is controlled by M phase specific molecular rearrangements in the nuclear pore complex (NPC). These changes allow a transport inhibitory nucleoporin, Nup53p, to bind the karyopherin Kap121p specifically during mitosis, slowing its movement through the NPC and inducing cargo release. Yeast strains that possess defects in the function of Kap121p or the fidelity of the inhibitory pathway are delayed in mitosis. We propose that fluctuations in Kap121p transport mediated by the NPC contribute to controlling the subcellular distribution of molecules that direct progression through mitosis.

Introduction

The separation of the cell's chromatin and transcriptional machinery from the cytoplasm demands that cells regulate a vast array of macromolecular traffic across the nuclear envelope (NE). The basic machinery that coordinates this transport has been largely described (see Macara, 2001; Weis, 2003). Cargo molecules are identified by soluble transport factors called karyopherins (or kaps, aka importins and exportins). The kaps then bind to nuclear pore complexes (NPCs) that form gateways across the NE barrier. A series of binding and release steps then ensue, which facilitates transport of the kap and its cargo through the NPC until terminated by the GTPase Ran. The ability of the nuclear transport system to accommodate multiple types of cargo arises from the diversity of the kaps, the majority of which belong to the β−karyopherin family. Each kap recognizes a distinct set of cargos based on the sequence or structure of a nuclear localization signal (NLS) or nuclear export signal (NES) on the cargo molecules. Importantly, the cargo sets recognized by individual kaps contain overlapping members, establishing a level of functional redundancy between certain karyopherins (Jans et al., 2000; Rout and Aitchison, 2001).

The NPC forms the transport channel across the NE. This complex structure is composed of ~30 proteins termed nucleoporins (or nups) (Rout et al., 2000; Cronshaw et al., 2002) that form repetitive substructures organized with 8-fold symmetry around an axis perpendicular to the NE and 2-fold pseudosymmetry parallel to the NE. Consistent with this organization, most nups can be detected on both the cytoplasmic and nucleoplasmic faces of the NPC. Built upon the symmetrical components of the NPC are asymmetric fibril structures that extend into the cytoplasm and the nucleoplasm (Rout and Aitchison, 2001).

Translocation through the NPC is initiated by the binding of the kap-cargo complex to a group of nups characterized by the presence of phenylalanine-glycine (FG) repetitive peptide motifs. The interactions between the FG-nups and the kaps are believed to facilitate the movement of the kap-cargo complex through the NPC. Several models have been proposed to explain this movement. The "selective phase" model argues that the FG-nups form a hydrophobic network that is selectively permeabilized by kaps (Ribbeck and Gorlich, 2001, 2002). Another proposes that the channel, surrounded by filamentous FG-nups, forms a "virtual gate" by entropically excluding molecules that cannot interact with the NPC (Rout et al., 2000). By interacting with the FG-nups, kaps overcome this barrier and progress through the channel. These models rely on the idea that kap-nup interactions are weak or rapidly dissociated (Gilchrist et al., 2002) in order to achieve rates of import sufficient to accommodate the huge flow of molecules across the NE (reviewed in Macara, 2001; Weis, 2003).

As a blueprint has emerged for the basic mechanisms of nuclear transport, so has the idea that transport can be regulated to orchestrate changes in nuclear physiology including gene transcription, DNA replication, and chromosome segregation. Well-documented examples of regulated transport utilize posttranslational modifications of cargo molecules (generally phosphorylation but also acetylation) to either inhibit or induce their transport by reducing or potentiating their interactions with kaps (reviewed in Kaffman and O'Shea, 1999; also see Madison et al., 2002).

A less explored concept is that nuclear transport can be regulated by altering the function of components of the transport machinery. This idea is supported by several observations that suggest changes in the NPC alter transport. For instance, alterations in the size of the NPC translocation channel have been detected during the cell cycle and in studies comparing quiescent and proliferating cells (Feldherr and Akin 1993, 1994). Moreover, in vitro import assays have been used to show that phosphorylation of transport proteins, most likely nups, can inhibit specific nuclear transport pathways (Kehlenbach and Gerace, 2000). The mechanistic bases for these effects are unclear.

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A more complex role for the NPC in modulating transport is also apparent from early observations demonstrating that mutations in an individual yeast nup could differentially affect the import of distinct cargos (Nehrbass et al., 1993). Subsequently, data showing that certain kaps preferentially or exclusively interact with specific nups led to the hypothesis that at least some kaps may follow distinct pathways through the NPC (Rout et al., 1997; Marelli et al., 1998) thus providing a potential site for regulation. Consistent with this idea, nups have been shown to preferentially bind to specific kaps. For example in yeast, Nup1p and Nup2p interact preferentially with Kap95p/Kap60p (Allen et al., 2001; Dilworth et al., 2001) and Nup53p interacts solely with Kap121p/Pse1p (Marelli et al., 1998). Similarly in vertebrates, Kap-β1 displays a preference for Nup153 over other FG-nups (Shah et al., 1998) and both Nup153 and Nup98 have M9-like NLSs recognized by Kap-β2 (Nakielny et al., 1999; Fontoura et al., 2000). In some cases, a link has been made between these interactions and the control of specific transport pathways (Shah and Forbes, 1998; Ullman et al., 1999; Marelli et al., 2001; Walther et al., 2001).

Here, we have defined a previously uncharacterized role for the NPC in regulating import controlled by the yeast kap, Kap121p. Our analysis reveals that the NPC can specifically inhibit the Kap121p-mediated import pathway during the mitotic phase of the cell cycle. The inhibitory mechanism is initiated during mitosis by structural rearrangements within the NPC that expose a masked binding site for Kap121p on Nup53p. Nup53p thus functions as a transport inhibitory nup, slowing the movement of Kap121p into the nucleus and inducing Kap121p to release its cargo. The significance of this mechanism is underscored by data showing that the regulation of Kap121p-mediated import plays a key role in progression through mitosis.

Results

Progression through Mitosis Is Delayed in *kap121* Mutants

Kap121p is an essential yeast karyopherin for which a limited number of imported cargo molecules have been identified. Inspection of the morphology of two temperature sensitive (ts) alleles, *kap121-34* and *kap121-41* (Marelli et al., 2001; Leslie et al., 2002; data not shown), revealed that cells grown at the permissive temperature contained a higher percentage of large-budded and multibudded cells as compared to their wild-type (WT) counterparts (data not shown) suggesting that there is a higher percentage of mitotic cells in the mutant cell population. Consistent with this idea, FACS analysis showed that the majority of *kap121-34* cells in culture contained a 2C or greater complement of DNA (Figure 1A). Furthermore, shifting cultures to the nonpermissive temperature (37°C) for 3 hr led to an increase in the number of mutant cells containing greater than 2C DNA content at 37°C (Figure 1A).

To further investigate the mitotic delay exhibited by the *kap121-34* mutant, we monitored levels of the mitotic cyclin Clb2p. Upon release from α factor-induced G1 arrest, levels of Clb2p rose at similar times in both the

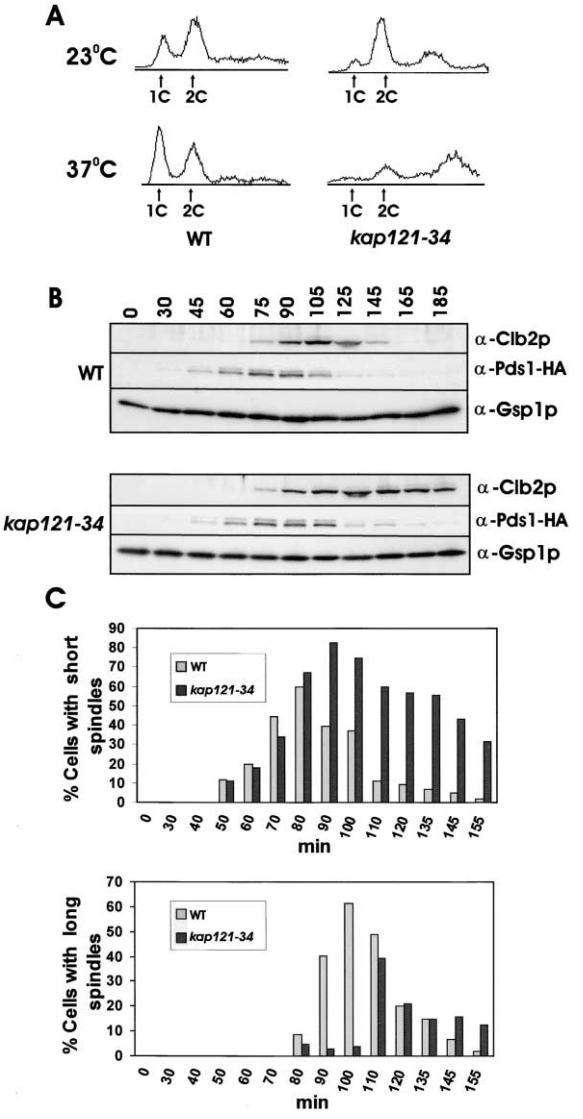

Figure 1. *KAP121* Mutant Strains Exhibit a Delay in Progression through Mitosis

(A) FACS analysis was performed on wild-type (WT) DF5 cells and a strain harboring the *kap121-34 ts* allele (KP121-34) grown at 23°C or 37°C for 3 hr. The positions of 1C and 2C peaks are indicated.

(B) Logarithmically growing cultures of DF5 (WT) and KP121-34 cells expressing a plasmid-borne copy of PDS1-HA were synchronized in G1 with α factor (time = 0 min). α factor was then removed and cells were grown at 23°C in YPD. Samples were taken at the indicated times and total cell lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting using antibodies directed against Clb2p (α-Clb2p), the HA tag, and a load control, Gsp1p (α-Gsp1p).

(C) Cultures of DF5 (WT) and the KP121-34 cells producing tubulin-GFP were arrested in G1, released, and scored for short (1–4 μm) or long (>4 μm) spindles at the indicated times.

WT and *kap121-34* cells suggesting that they progressed into mitosis at similar rates. However, the length of mitosis was extended in the *kap121-34* mutant as the levels of Clb2p remained elevated for a significantly longer period of time than in WT cells (Figure 1B). This mitotic delay appears to be initiated at a point near the metaphase-anaphase transition. We observe that Pds1p

degradation, an APC substrate eliminated at this point, was not affected in the *kap121-34* mutant suggesting that cells normally approach this transition. However, an examination of spindle length following G1 release showed that *kap121-34* cells were delayed in their transition from short spindles (1–4 μm) to long spindles (>4 μm; Figure 1C), consistent with a mitotic delay beginning with progression from metaphase to anaphase.

M Phase Specific Regulation of Kap121p-Mediated Transport

On the basis of the cell cycle defects observed in the *kap121* mutants, we hypothesized that Kap121p plays a role in mediating the flux of mitotic regulators into the nucleoplasm. We envisioned two scenarios by which this could occur. In one case, nuclear levels of Kap121p cargo molecules could be regulated by either their levels of synthesis or ability to bind Kap121p. Alternatively, the import of Kap121p specific cargos could be controlled by altering the rate of Kap121p entry, and thus its cargo, into the nucleus. In this latter case, global changes in Kap121p-mediated import are predicted to accompany progression through mitosis. To address these two possibilities, we examined the levels of Kap121p-mediated import at various stages of the cell cycle using a GFP-tagged reporter cargo. Cells containing the reporter were synchronized in G1 phase using α factor, in S phase using hydroxyurea, or in G2/M phase using nocodazole, and the steady-state distribution of the reporter was examined by fluorescence microscopy. The import of two separate Kap121p-specific import reporters was examined, a Pho4-NLS-GFP protein containing the NLS of the Pho4 transcription factor (Kaffman et al., 1998) and a Ste12-NLS-GFP fusion containing the C terminus of the Ste12p transcription factor (Leslie et al., 2002). Both reporters were concentrated in the nucleus of cells arrested in S (Figure 2A) and G1 phase (data not shown). In contrast, these reporters failed to accumulate in the nucleus and were distributed throughout the cell in cultures arrested in G2/M (Figure 2A). This same distribution pattern was also visible in a *cdc15-2* mutant (Hartwell, 1971) arrested in telophase at the nonpermissive temperature (Figure 2B). These changes in import were not due to alterations in the stability or abundance of the reporters during the cell cycle (data not shown). For comparison, import was also monitored with various other reporters including those recognized by Kap95p/Kap60p (cNLS-GFP), Kap104p (RG-NLS; Lee and Aitchison, 1999), and Kap123p (rpL25-NLS; Rout et al., 1997), each of which continued to accumulate in nuclei of G2/M arrested cells (Figure 2A).

The inability of Kap121p cargo reporters to accumulate in the nucleus could be caused by a reduced binding to Kap121p. For example, the phosphorylation of Pho4p is responsible for the phosphate ion dependent inhibition of Pho4p binding to Kap121p (Kaffman et al., 1998). The Pho4-NLS-GFP reporter used here is constitutively imported and not affected by phosphate ion levels; however, the Pho4-NLS does contain several potential phosphorylation sites. To determine whether phosphorylation plays a role in inhibiting its import during mitosis, we constructed an NLS that lacked phosphorylation sites by replacing each of the serine and threonine resi-

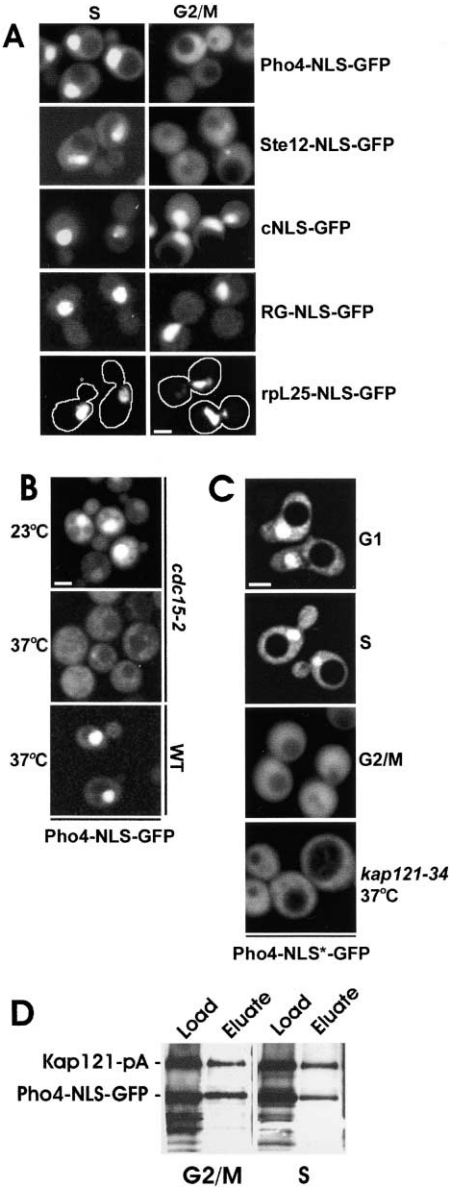

Figure 2. Kap121p-Mediated Import Is Inhibited during Mitosis

(A, B, and C) DF5 cells transformed with the plasmid pPHO4-NLS-GFP, pSTE12-NLS-GFP, pcNLS-GFP, pRG-NLS-GFP, pRPL25-NLS-GFP, or pPHO4-NLS*-GFP were grown to early log phase and arrested in G1 with α factor, S with hydroxyurea, or G2/M with nocodazole as indicated. Strains harboring ts alleles of *CDC15* (cdc15-2B) or *KAP121* (KP121-34) and containing pPHO4-NLS-GFP or pPHO4-NLS*-GFP, respectively, were grown at 23°C, and then shifted to 37°C for 3 hr. Note, only KP121-34 cells grown at 37°C are shown in (C). The subcellular distribution of the GFP reporters was examined using fluorescence confocal microscopy. Bars are equal to 5 μm.

(D) A yeast strain producing a protein-A tagged Kap121p chimera (KP121pA) and PHO4-NLS-GFP were arrested in S or G2/M phase. Kap121-pA was affinity purified from postribosomal supernatants using IgG Sepharose beads. Proteins of the postribosomal supernatant (Load) and the eluate of the IgG Sepharose beads were separated by SDS-PAGE and Western blotted using α-GFP antibodies to detect Pho4-NLS-GFP. The HRP-conjugated secondary antibodies also bind the protein-A moiety in Kap121-pA.

dues with alanines (Pho4-NLS*). This mutant NLS was functional and its nuclear import was dependent on Kap121p (Figure 2C). When examined in nocodazole-arrested cells, the import of Pho4-NLS*-GFP was inhibited (Figure 2C) demonstrating that phosphorylation of the reporter does not play a role in blocking its import. Furthermore, we failed to detect a change in the binding of Kap121p to the Pho4-NLS in G2/M phase-arrested cells, with similar amounts of the Pho4-NLS-GFP reporter being bound to Kap121-pA affinity-purified from cells arrested in G2/M or S phase (Figure 2D). These results support the idea that M phase import inhibition is not caused by the inability of Kap121p to bind its cargo.

We concluded that the M phase specific defect in Kap121p-mediated import was likely due to an inhibition of the translocation of Kap121p through the NPC. To test this idea, we took advantage of previous reports showing that the treatment of cells with metabolic poisons such as 2-deoxyglucose, reduce cellular levels of RanGTP (Schwoebel et al., 2002). Because nuclear RanGTP is not required for the translocation of at least certain import kaps through the NPC (Kose et al., 1997; Ribbeck et al., 1999; Englmeier et al., 1999; Ribbeck and Gorlich, 2001; Schwoebel et al., 2002), but it is required for their export back to the cytoplasm, treatment with 2-deoxyglucose can lead to kap accumulation in the nucleus (Schwoebel et al., 2002). With these observations in mind, we specifically monitored the import step of the Kap121p transport cycle by examining the subcellular distribution of Kap121-GFP in G1, S, and G2/M phase-arrested cells following treatment with 2-deoxyglucose. Treatment of cells with 2-deoxyglucose rapidly (within 15 min) caused an accumulation of Kap121-GFP in the nucleus of cells arrested in G1 (Figure 3A) and S phase (data not shown). By comparison, G2/M-arrested cells showed little or no nuclear accumulation of Kap121-GFP during the same time period (Figure 3A). We quantified these results using an approach similar to that previously described by Shulga et al. (1996) for determining relative rates of nuclear transport. Arrested cells were treated for various times with 2-deoxyglucose and scored for the nuclear accumulation of Kap121-GFP. In G1 arrested cells, 2-deoxyglucose treatment induced a rapid nuclear accumulation of Kap121-GFP with 50% of the cells showing a nuclear signal within 13 min (Figure 3B). In comparison, G2/M-arrested cells showed a much slower rate of nuclear accumulation requiring 35 min to reach similar levels (Figure 3B). These results suggest that a reduced rate of Kap121p translocation through the NPC accounts for the inhibition of transport during M phase.

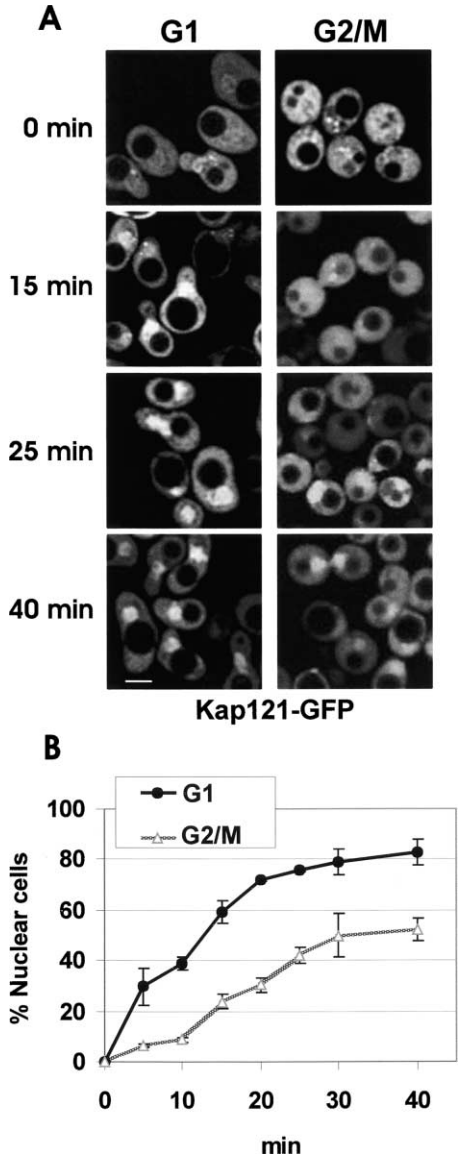

Figure 3. Movement of Kap121p into the Nucleus Is Inhibited in G2/M-Arrested Cells

(A) DF5 cells containing the plasmid pKAP121-GFP were arrested in G1 with α factor or G2/M with nocodazole (0 min). Cells were then washed and placed in media lacking glucose and containing 100 mM 2-deoxyglucose. The distribution of Kap121-GFP was documented by fluorescence confocal microscopy at the indicated times. Bar is equal to 5μm.

(B) In a similar experiment, cells were scored for nuclear fluorescence at various times after addition of 2-deoxyglucose using a confocal microscope. The percentage of cells showing nuclear fluorescence was plotted against time.

Binding of Kap121p to the NPC Is Altered during Mitosis

The decreased entry of Kap121p into the nucleus during mitosis is likely explained by changes in its interactions with the NPC. Two possible mechanisms were envisioned. First, lowering the affinity of Kap121p for nups could restrict its entry into the NPC. Alternatively, exposure to high affinity binding sites could inhibit movement through the translocation channel. Since we did not detect a global change in import, but rather only that mediated by Kap121p, the latter mechanism would likely invoke a nup that interacts specifically with Kap121p. One candidate was Nup53p. Previous work has shown that Nup53p isolated from asynchronous cell cultures is specifically associated with Kap121p (Marelli et al., 1998). However, by the nature of these experiments it was unclear whether this interaction varied during the cell cycle. To address this question, protein A tagged versions of Kap121p or Nup53p were purified from cell cultures arrested in S or G2/M phase. Strikingly, these

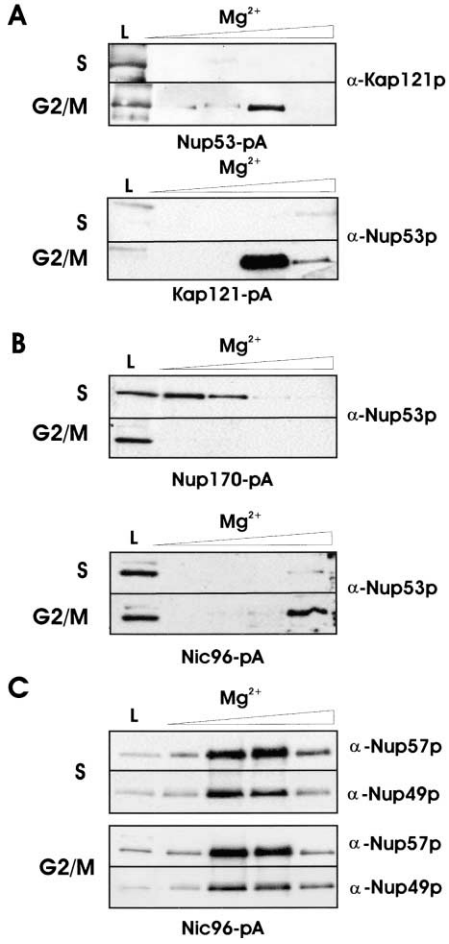

Figure 4. Molecular Rearrangements within the NPC during Mitosis

(A, B, and C) Cell extracts were prepared from yeast strains producing protein A-tagged Nup53p, Kap121p, Nup170p, and Nic96p (NP53pA, KP121pA, NP170pA, and NIC96pA) arrested with hydroxyurea (S) or nocodazole (G2/M). The protein A fusions were affinity purified using IgG-Sepharose beads. The beads were washed and eluted with a step gradient of 200, 500, 1000, and 2000 mM $MgCl_2$. Eluted proteins were analyzed by SDS-PAGE and Western blotting using α-Nup53p, α-Kap121p, α-Nup57p, or α-Nup49p antibodies. Lane L contains a sample of the fraction loaded on the IgG-Sepharose.

experiments revealed changes in the association of Nup53p with Kap121p during the cell cycle. We failed to detect an interaction between Nup53p and Kap121p in S (Figure 4A) or G1 phase- (data not shown) arrested cells following purification of either tagged protein. In contrast, a clear association between these proteins was detected following their purification from G2/M-arrested cells (Figure 4A).

The change in the interaction of Nup53p with Kap121p during the cell cycle was accompanied by alterations in its association with neighboring nups. Using affinity purification techniques like those described above, we observed that the association of Nup53p with Nup170p could be detected in S- and G1-arrested cells, but not in cells arrested in G2/M (Figure 4B, see also Iouk et al., 2002). Similarly, we failed to detect an interaction between Nup53p and Nup170p in cdc15-2B cells arrested at the nonpermissive temperature in telophase (data not shown). Conversely, Nup53p was tightly bound to purified Nic96p in M phase-arrested cells, but only trace amounts of Nup53p were detected in S phase-arrested cells (Figure 4B). Similar molecular rearrangements were not uniformly detected among nucleoporins during the cell cycle. For example, the associations of Nic96p with Nsp1p, Nup57p, and Nup49p were not altered (Figure 4C). These results suggest that specific molecular rearrangements occur in the NPC during M phase that allow Nup53p to interact with Kap121p and Nic96p.

Nup53p Functions to Inhibit Kap121p-Mediated Import during Mitosis

The association of Kap121p with Nup53p during M phase supports the hypothesis that Kap121p-mediated import is inhibited by its interaction with Nup53p. This suggests that mutations in Nup53p that fail to bind Kap121p would increase the rate of transport during M phase. We therefore examined both the uptake of Kap121p into the nucleus and import of the Pho4-NLS-GFP reporter during M phase in a strain where Nup53p was replaced with a deletion mutant (nup53-Δ405-430) lacking the 26 amino acid residue Kap121p binding domain (KBD) (Lusk et al., 2002). Cells expressing the *nup53-Δ405-430* mutation were arrested in G2/M phase and the nuclear accumulation of Kap121p was then induced using 2-deoxyglucose. As shown in Figures 5A and 5B, deletion of the KBD from Nup53p stimulated uptake of Kap121p into the nucleus during mitosis as compared to WT cells. Consistent with these results, removal of the inhibitory effects of the Nup53p KBD also resulted in the steady-state accumulation of the Pho4-NLS-GFP reporter in the nucleus of G2/M synchronized cells (Figure 5C). Of note, import stimulation, albeit to a lesser extent, was also observed in the *nup53* null strains (data not shown). These results establish a clear role for Nup53p as an inhibitor of Kap121p-mediated import during mitosis.

We have previously shown that the KBD of Nup53p shares sequence similarity with Kap121p-specific NLSs (Lusk et al., 2002). This similarity suggests the KBD may bind to the NLS binding region of Kap121p. This model predicts that the Nup53p KBD may stimulate the release of cargo from Kap121p by competing for NLS binding. To test this, we assembled a recombinant Kap121p/Pho4p complex by incubating Kap121p with immobilized GST-Pho4p. After washing to remove free Kap121p, the Kap121p/Pho4p complex was released from GST (Figure 5D, lane L) and presented to bead bound GST-Nup53p. We detected that the majority of Kap121p was bound to Nup53p, while Pho4p was detected exclusively in the unbound fraction (Figure 5D, lane FT). In contrast, a deletion mutant of Nup53p lacking the KBD (GST-1-375) failed to alter the Kap121p/Pho4p complex. These results are consistent with a model in which Nup53p also inhibits cargo binding to Kap121p during mitosis.

Increased Levels of Nup53p Inhibit Progression through Mitosis

As discussed above, masking of the Nup53p KBD from Kap121p is likely controlled by interactions with Nup170p

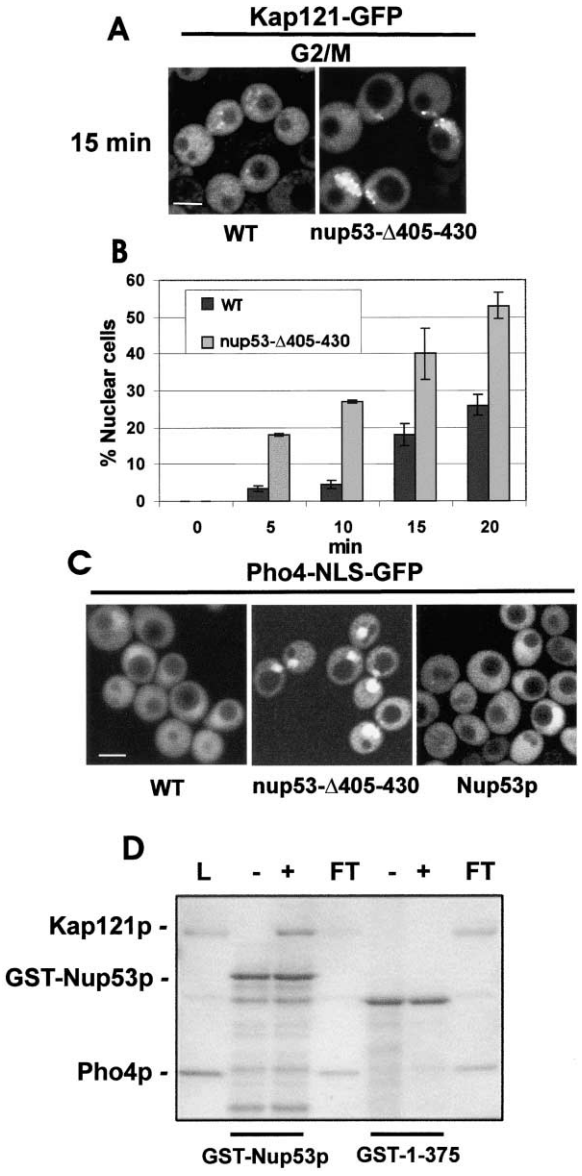

Figure 5. The KBD of Nup53p Is Required for Kap121p-Mediated Import Inhibition during Mitosis

(A and B) The localization of Kap121-GFP in DF5 (WT) cells and a *nup53*Δ strain (NP53-Δ405-430) containing a plasmid-encoded Nup53p deletion lacking the KBD (nup53-Δ405-430) was examined following G2/M arrest with nocodazole and treatment for 15 min with 100 mM 2-deoxyglucose. The distribution of Kap121-GFP was documented using a confocal microscope. Nuclear uptake of Kap121-GFP was quantified (B) at the indicated times as described in Figure 3B.

(C) The distribution of the Pho4-NLS-GFP reporter in the strains NP53-Δ405-430 (nup53-Δ405-430), NP53-FL53 (*nup53*Δ strain containing a plasmid copy of *NUP53*; Nup53p), and DF5 (WT) was examined after G2/M arrest with nocodazole. Bars are equal to 5μm.

(D) GST-tagged Nup53p or a truncation of Nup53p lacking the KBD (GST-1-375) were synthesized in and purified from *E. coli*. Five μg of each fusion was immobilized on GT-Sepharose beads. The beads were then incubated with (+) or without (−) ~10 μg of a preformed complex of Kap121p and Pho4p (L). After washing, the bound fraction was eluted with SDS-PAGE sample buffer. Equivalent amounts of the bound and flow through (FT) fractions were analyzed by SDS-PAGE and proteins were visualized by Coomassie blue staining.

(Figure 4B; Lusk et al., 2002). A prediction of this model is that an increase in the levels of Nup53p that exceed the binding capacity of Nup170p would constitutively inhibit import and perhaps cause similar cell cycle defects to those noted in the *kap121-34* ts strain (Figure 1). In fact, increased levels of Nup53p have been shown previously to inhibit import of Kap121p cargo (Marelli et al., 2001). We tested whether moderate increases in the levels of Nup53p would also delay progression through mitosis. For these experiments, *NUP53* under the control of the *CUP1* promoter was introduced into a *nup53*Δ mutant strain. In the absence of induction, this construct produces approximately 5- to 10-fold higher amounts of Nup53p than the endogenous levels (see Figure 1C of Marelli et al., 2001). As shown in Figures 6A and 6B, under these conditions of moderate *NUP53* overexpression, the cell population displayed increased numbers of cells containing a 2C complement of DNA and a higher percentage of large-budded cells as compared to a WT counterpart. Moreover, a strain overproducing the nup53-Δ405-430 deletion construct, lacking the KBD, showed a normal distribution of DNA complement and budding index. An examination of Clb2p levels (Figure 6C) and spindle morphology (data not shown) in Nup53p overproducing cells also mimicked the *kap121-34* mutant cell cycle phenotypes described above. The delay observed in the degradation of Clb2p was not a function of the inner nuclear membrane proliferation previously observed in cells massively overproducing Nup53p (Marelli et al., 2001), as similar results were obtained upon overproduction of a Nup53p C-terminal truncation that contains the KBD and targets to the NPC, but fails to induce membrane proliferation (nup53-1-448; Figure 6C).

Discussion

Here, we describe a mechanism for regulating nuclear import that is controlled by the NPC. While it is generally accepted that the regulation of nucleocytoplasmic transport in response to environmental cues or progression through the cell cycle can be controlled by modifications of a cargo, which alter binding to its cognate karyopherin, our data demonstrate that cell cycle changes in the NPC itself can regulate specific transport pathways. In particular, we show that Nup53p acts as a transport inhibitory nucleoporin (iNup), slowing nuclear transport mediated by Kap121p during mitosis. These results establish that the NPC plays a more elaborate role in mediating nucleocytoplasmic transport than previously appreciated.

Kap121p-mediated import is active in interphase but is inhibited during mitosis. This is in contrast to import mediated by several karyopherins tested here, including the Kap60p/Kap95p complex, Kap123p, and Kap104p, which appear unaltered during the cell cycle. A decrease in the steady-state nuclear levels of Kap121p cargo was observed at the G2/M transition (in nocodazole-arrested cells, Figure 2) and into telophase (in arrested cdc15-2B cells, Figure 2B). This decreased nuclear accumulation could not be attributed to changes in cargo binding to Kap121p (Figures 2C and 2D) or in the permeability of the NE as measured using diffusion assays (data not

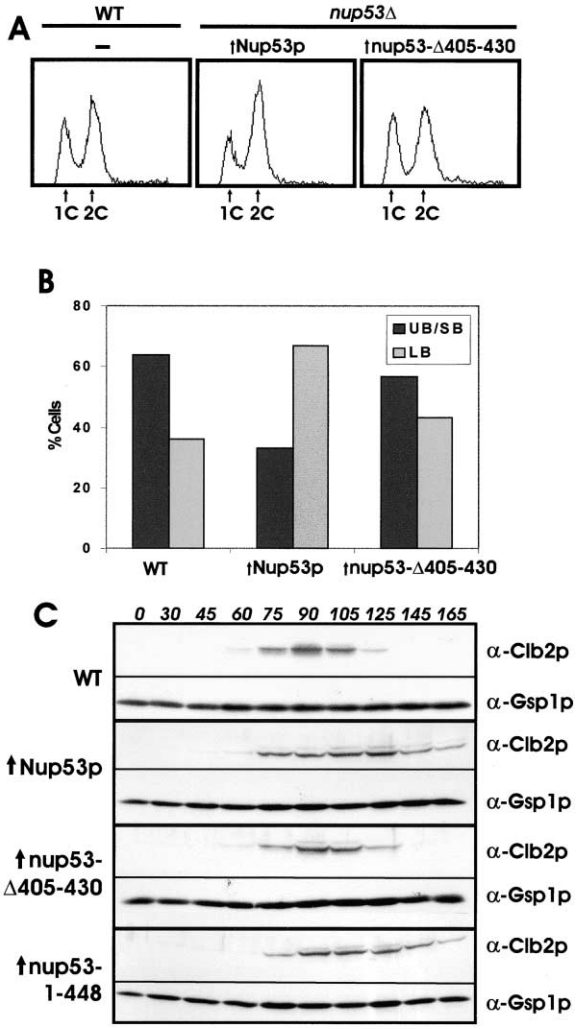

Figure 6. Increased Levels of Nup53p Delay Progression through Mitosis

(A) FACS analysis was performed on logarithmically growing cultures of DF5 (WT) cells and *nup53*Δ strains containing plasmid-encoded *NUP53* (NP53-FL53) or *nup53-Δ405-430* (NP53-Δ405-430) and producing moderately increased levels of Nup53p (↑Nup53p) or nup53-Δ405-430 (↑nup53-Δ405-430), respectively. The positions of 1C and 2C peaks are indicated.

(B) The morphologies of cells in these cultures were also examined and the percentage of large budded (LB) cells was compared to the combined percentage of unbudded and small budded (UB/SB) cells.

(C) Logarithmically growing cultures of DF5 cells containing an empty plasmid (WT), NP53-FL53 cells (↑Nup53p), NP53-Δ405-430 cells (↑nup53-Δ405-430), and NP53-1-448 (↑nup53-1−448) were arrested with α factor in G1 (time = 0 min). α factor was then removed and cells were grown at 23°C in YPD. Samples were taken at the indicated times (min) and total cell lysates were prepared. Proteins were separated by SDS-PAGE and analyzed by Western blotting using antibodies directed against Clb2p (α-Clb2p) and a load control, Gsp1p (α-Gsp1p).

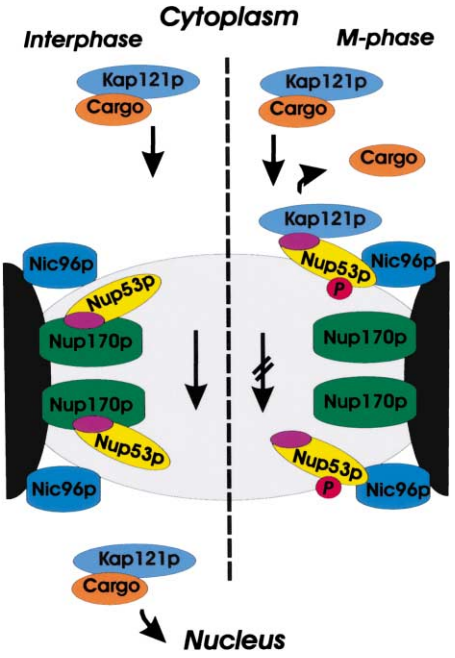

Figure 7. A Model for the Cell Cycle Regulation of Kap121p-Mediated Transport by Nup53p

Kap121p-mediated transport is proposed to proceed through interactions with FG-nups (represented in gray). During interphase Nup53p is bound to Nup170p, which masks the KBD of Nup53p and prevents its interaction with Kap121p. As cells enter M phase, Nup53p is phosphorylated, is no longer bound to Nup170p, and is detected in complex with Nic96p. Release from Nup170p exposes the KBD (shown in purple) and allows Kap121p to bind to Nup53p. We propose that the KBD is a high-affinity Kap121p binding site and, therefore serves to slow Kap121p's translocation through the NPC. In addition, competition between the KBD and a cargo NLS results in the premature release of cargo from Kap121p.

shown). Rather, import inhibition is apparently due to a decrease in the efficiency of Kap121p translocation through the NPC (Figure 3).

An obvious candidate for controlling the altered transport of Kap121p was Nup53p. We have previously shown that Nup53p binds specifically to Kap121p (Marelli et al., 1998) and we identified a region within Nup53p resembling a Kap121p-specific NLS (Lusk et al., 2002) responsible for this interaction. This region of Nup53p is contained within a domain that also directly interacts with a neighboring nup, Nup170p. Thus, binding to Nup170p prevents Nup53p from interacting with Kap121p (Lusk et al., 2002 and C.P.L. and R.W.W., unpublished data). Analysis of this molecular switch revealed the surprising observation that during interphase and active Kap121p transport, most of Nup53p is associated with Nup170p, not Kap121p. Whereas during mitosis, when Kap121p transport is blocked, Nup53p is tightly associated with Kap121p but remains anchored to the NPC through Nic96p. These data suggest the counterintuitive model in which Kap121p binding to Nup53p inhibits transport; rather than promoting it (Figure 7). Indeed, this idea is consistent with previous data showing that *nup53*Δ mutants do not block the nuclear accumulation of Kap121p cargo and that import inhibition can be seen upon overproduction of Nup53p (Marelli et al., 2001), a circumstance that would create a pool of Nup53p free of Nup170p.

The specific events that trigger molecular rearrangements within the NPC are unclear, but Nup53p release from Nup170p presumably frees the Kap121p binding domain (KBD) allowing Nup53p to bind tightly to

Kap121p (Figure 4A), inhibiting transport. This is supported by the striking observation that the removal of the KBD from Nup53p relieves the M phase specific import inhibition (Figure 5C). Furthermore, it is likely that the phosphorylation of Nup53p (Marelli et al., 1998) and potentially other nups are a key step in this process. At present, it is unknown precisely how this modification affects Nup53p's association with its neighboring nups; however, phosphorylation does not appear to influence its binding to Kap121p (Marelli et al., 1998, T.M., C.P.L., and R.W.W., unpublished data). These data reinforce the idea that the binding of Nup53p to Kap121p during mitosis is controlled by the accessibility of the KBD.

To derive an explanation for how these binding events control Kap121p transport, one must first consider current models for transport through the NPC. It is believed that the kap binding FG-nups, including Nup53p, line the translocation channel through the NPC, forming an estimated 160 kap binding sites per pore (Rout et al., 2000). Models explaining the movements of kaps through this sea of FG repeat domains (see Introduction) rely on the assumption that kap-FG-nup interactions must be of low affinity and/or rapidly dissociated to achieve reasonable rates of translocation through the channel. Indeed, the K_D of kap-FG-nup interactions has been measured in the micromolar range (Bayliss et al., 1999, Pyhtila and Rexach, 2003). In contrast, NLS interactions with kaps are of higher (nanomolar K_D) affinities (Gilchrist et al., 2002). The structural similarity of the Nup53p KBD to the Kap121p NLS sequence (Lusk et al., 2002) suggests that this interaction is more similar to a kap-cargo interaction than a kap-FG repeat interaction, and thereby, presumably of higher affinity. Consistent with this idea, it is our empirical observation that the association of Nup53p with Kap121p is stronger than that between Kap121p and other FG-nups, as the latter group can be dissociated from Kap121p under conditions that do not affect the interaction of Nup53p with Kap121p (C.P.L., T.M., and R.W.W., unpublished data).

In addition to slowing the rate of Kap121p movement through the NPC during mitosis, our data suggest a second factor contributing to import inhibition is the release of cargo stimulated by competition with Nup53p for the NLS binding site on Kap121p (Figure 5D). Since Nup53p is positioned on both the cytoplasmic and nucleoplasmic faces of the NPC (Marelli et al., 1998; Rout et al., 2000), exposure of the Kap121p/cargo complex to cytoplasmically disposed Nup53p could induce cargo release prior to translocation through the NPC (Figure 7). A nup-induced cargo release mechanism has also been proposed for the release of cargo from Kapβ2/transportin upon binding to an M9 NLS site in mammalian Nup98 (Fontoura et al., 2000). In this case, however, the localization of Nup98 to the nuclear side of the NPC suggests that this represents a terminal step in the release of cargo into the nucleoplasm. Moreover, both mammalian Nup153 and yeast Nup1p form particularly stable interactions with Kap-β1/importin β (Shah et al., 1998) and Kap95p (Pyhtila and Rexach, 2003), respectively. These nups are also localized exclusively on the nucleoplasmic face of the NPC, which has led to the proposal that they facilitate transport by acting as high affinity sites that draw these kaps away from low-affinity FG sites lining the channel. Thus, the functions of Nup153, Nup98, and Nup1p as potential facilitators of transport and Nup53p as an iNup may be governed by their localization within the NPC.

Having uncovered a mechanism for regulating the entry of Kap121p into the nucleus during mitosis, we are faced with the question of what role Kap121p-mediated import plays in cell cycle progression. We have shown that reduced import caused by the *kap121* ts alleles or overproduction of Nup53p delays progression through mitosis beginning at or following metaphase (Figures 1 and 6). We surmise that, since levels of Kap121p-mediated import are normally reduced during mitosis (Figure 2), constitutive defects in Kap121p import may produce a mitotic phenotype by interfering with the nuclear influx of cargos occurring at transition points prior to and/or near the end of mitosis. Multiple Kap121p cargos are likely affected, one of which is Cdh1p, a nonessential protein that binds the anaphase-promoting complex (APC/C) and mediates the degradation of Clb2p (see Zachariae and Nasmyth, 1999). Cdh1p is localized to the cytoplasm during mitosis but accumulates in the nucleus late in mitosis where it is detected during G1 (Jaquenoud et al., 2002). The nuclear accumulation of Cdh1p during G1 is dependent on Kap121p and is inhibited in *kap121* ts mutants (Jaquenoud et al., 2002) and cells overexpressing *NUP53* (data not shown).

The inhibition of Kap121p import during mitosis likely contributes to reducing the nuclear concentration of multiple Kap121p cargo molecules. Which cargos are normally targeted by this regulation, and the specific role that this transport regulation plays in mitosis, however, remain unclear. Defining these functions is complicated by the observations that cells lacking *NUP53* or expressing the *nup53-Δ405-430* deletion, conditions that clearly relieve Kap121p import inhibition (Figure 5), do not exhibit a clear defect in progression through mitosis. These observations are likely explained by two factors, (1) multiple, and often redundant, transport pathways control cargo localization and (2) cargos themselves exhibit redundant functions. The distribution of cargos between the nucleus and the cytoplasm is often controlled by the concerted action of both import and export kaps (Weis, 2003), where the steady state is achieved based on the relative activities of the two pathways. A case in point is again Cdh1p, whose localization is controlled by Kap121p (import) and Msn5p/Kap142p (export) (Jaquenoud et al., 2002). We predict that the inhibition of Kap121p import and active export contribute to excluding Cdh1p from the nucleus until the later stages of mitosis. This functional overlap may explain the sensitivity to overexpression of *CDH1* exhibited by both *nup53Δ* and *msn5Δ* strains (Supplemental Figure S1A available at http://www.cell.com/cgi/content/full/115/7/813/DC1; Jaquenoud et al., 2002).

Several other results also implicate the control of Kap121p import in M phase progression. We have detected functional interactions between Nup53p and Bub2p, one of a group of proteins functioning in the mitotic exit network (see Jensen and Johnston, 2002), with double-null strains exhibiting heightened sensitivity to microtubule destabilizing drugs (Supplemental Figure S1B available on *Cell* website). In addition, previously unexplained data, including the synthetic lethal interactions of *NUP170* and *SIC1* (an inhibitor of the Clb2p/

Cdc28p complex) null mutants and the suppression of a *cdc14* mutant by overexpression of *KAP121* (Yuste-Rojas and Cross, 2000) further underscore a role for the Nup53p transport inhibitory mechanism during mitosis. Fully understanding the functions of this pathway will require defining the repertoire of Kap121p cargos, understanding functional redundancy with other transport pathways, including others linked to mitotic progression (Asakawa and Toh-e, 2002; Shou and Deshaies, 2002), and integrating this information with the known, and often redundant, functions their cargos play in regulating mitosis.

In conclusion, our data demonstrate the existence of a previously undefined mechanism for regulating nucleocytoplasmic transport. This mechanism involves a direct role for the NPC, through cell-cycle specific changes in its molecular organization, in regulating specific karyopherin movements and participating in cell-cycle control events. While our experiments have focused on yeast, it seems likely that similar NPC-mediated regulatory events also function in higher eukaryotes, perhaps at points prior to NE disassembly or shortly after its reformation, as well as at other points in the cell cycle. This latter idea is stimulated by previous observations in mammalian cells showing that a subset of nups, including Nup153, Nup214, and Nup358, are phosphorylated during S phase when the NE is intact (Favreau et al., 1996), as well as data showing that phosphorylation, most likely of nups, can inhibit nuclear transport (Kehlenbach and Gerace, 2000).

Experimental Procedures

Yeast Strains and Media

Yeast strains were grown at 30°C unless otherwise indicated in YPD or in synthetic media (SM) supplemented with the appropriate nutrients and 2% glucose (Sherman et al., 1983). Yeast transformations were performed as described by Delorme (1989). The following strains were used in this study: DF5 (*Mat a ura3-52 his3-Δ200 trp1-1 leu2-3, 112 lys2-801*), NP53-A2, NP53pA, cdc15-2B (Marelli et al., 1998), NP170pA (Aitchison et al., 1995), KP121pA (Rout et al., 1997), KP121-34 (Leslie et al., 2002), NIC96pA (Rout et al., 2000), and BY4742 (*Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), ymr153wΔ (*nup-53Δ::KAN* in BY4742), ydr335wΔ (*msn5Δ::KAN* in BY4742), and ymr055cΔ (*bub2Δ::KAN* in BY4742) (Giaever et al., 2002). DF5BY, NP53-C1, BUB2-C1, and NP53/RGBUB2 are segregants from a cross between NP53-A2 and RGBUB2. pYNP53, pYΔ405-430, and pY1-448 were transformed into NP53-A2 to produce NP53-FL53, NP53-Δ405-430, and NP53-1-448, respectively.

Plasmids

The following plasmids were used in this study: pPHO4-NLS-GFP (EBO836) (Kaffman et al., 1998; provided by E. O'Shea, University of California, San Francisco, San Francisco, CA); pSTE12-NLS-GFP (pSte12p (aa 494–688)-GFP) (Leslie et al., 2002); pcNLS-GFP (Stade et al., 1997); pKAP121-GFP (pPS1069) (Seedorf and Silver, 1997; provided by P. Silver, The Dana Farber Cancer Institute, Boston, MA); pRG-NLS-GFP (Lee and Aitchison, 1999); pRPL25-NLS-GFP (provided by M. Rout, Rockefeller University, New York, NY); pYEX-BX (Clontech); pYΔ405-430, pYNP53, pY1-448, pGNP53, pG1-375 (Lusk et al., 2002); pGEX-KAP121 (Marelli et al., 1998); pGFP-TUB1 (provided by N. Adames, University of Alberta, Edmonton, Alberta, Canada); and pPDS1-HA (provided by M. Basrai, National Institutes of Health, Bethesda, MA).

PHO4 and *CDH1* ORFs were amplified from yeast genomic DNA and the resulting products were inserted into pGEX-6P-1 (Amersham Biosciences) or pGFP-C-fus (Niedenthal et al., 1996) to construct pGPHO4 and pCDH1-GFP, respectively. To construct pPHO4-NLS*-GFP, two complementary oligonucleotides were designed to encode a mutant of the Pho4p NLS by replacing all serine and threonine residues with alanines (**AANKVAKNKANAA**PYLNKRRGK PGPD**A**, changed residues in bold). The oligonucleotides were annealed and the resulting dsDNA was ligated into pPHO4-NLS-GFP, replacing the Pho4p NLS.

Procedures for Cell Cycle Arrest

Cells were grown in YPD to early log phase and treated with (1) 5 μg/ml α-factor (Sigma-Aldrich) for 2 hr to arrest in G1 phase; (2) 100 mM hydroxyurea (Sigma-Aldrich) for 2.5 hr to arrest in S phase; or (3) either 15 μg/ml nocodazole (Sigma-Aldrich) or 30 μg/ml benomyl (Sigma-Aldrich) for 2.5 hr to arrest in G2/M (Hardwick and Murray, 1995). Arrests were confirmed visually by microscopy or by FACS analysis. To arrest the cdc15-2B strain in late mitosis, cells were shifted from 23°C to 37°C for 3 hr (Hartwell, 1971; Marelli et al., 1998).

Fluorescence Microscopy

Yeast strains harboring the plasmids pGFP-TUB1, pKAP121-GFP, pPHO4-NLS-GFP, pPHO4-NLS*-GFP, pcNLS-GFP, pRPL25-NLS-GFP, pRG-NLS-GFP, or pSTE12-NLS-GFP were grown to early log phase in SM media and then transferred to YPD for 4 hr prior to cell cycle arrest. Cells were viewed directly using a Zeiss LSM 510 laser scanning confocal microscope (LSM 510 META; Zeiss MicroImaging, Inc.) to image the GFP fusion proteins. All images are 0.7 μm optical sections.

Budding Profiles, Spindle, and FACS Analysis

DF5 and KP121-34 strains were grown to early log phase at 23°C. Cultures were split, half were shifted to 37°C, and half retained at 23°C. Samples were taken after 3 hr and fixed in 3.7% formaldehyde to determine budding profiles or prepared for FACS analysis. In a similar experiment, DF5, NP53-FL53, and NP53-Δ405-430 strains were grown to an OD_{600} of 0.4 and also prepared as above. Cells were sonicated briefly and scored as single/small budded cells (less than 50% of the diameter of the mother cell), large budded, or multibudded. 300 cells were counted for each trial ($n = 3$). FACS analysis was performed as previously described (Basrai et al., 1996). Data were collected on a FACS Scan (Becton Dickinson, San Jose, CA) and analyzed using CellQuest.

Spindle length was evaluated at 23°C in DF5 and KP121-34 strains transformed with pGFP-TUB1 following release from α factor arrest as described in Harvey and Kellogg, (2003). Percentages of short or long spindles were determined from 150–200 cells per time point.

Kap121-GFP Import Assay

Similar to the protocol described in Shulga et al. (1996), DF5 and NP53-Δ405-430 strains transformed with pKAP121-GFP were used to evaluate the rate of Kap121-GFP import at different points in the cell cycle. Logarithmically growing cells were arrested as described above. Cells were then collected by centrifugation, washed twice with YPD lacking glucose (YP), and resuspended in YP containing 100 mM 2-deoxyglucose (Sigma-Aldrich). Images were acquired at different time points after addition of 2-deoxyglucose at 23°C. To evaluate relative rate differences in Kap121-GFP import, cells were scored for nuclear accumulation of fluorescence and represented graphically as the percentage of cells showing a nuclear signal versus time.

Isolation of Protein A Chimeras, Production of Cell Lysates, and Immunoblotting

250 ml cultures of yeast strains synthesizing protein-A (pA) fusion proteins (NP53pA, KP121pA, NP170pA, NIC96pA, and KP121pA transformed with pPHO4-NLS-GFP) were arrested as described above. Isolation of protein A fusions from cell lysates using IgG Sepharose (Amersham Biosciences) chromatography was performed as previously described (Marelli et al., 1998; Lusk et al., 2002). Following isolation, bead bound complexes were washed extensively with lysis buffer followed by two additional washes in lysis buffer containing 50 mM $MgCl_2$. Bound proteins were then eluted with a step gradient of $MgCl_2$ (200 mM, 500 mM, 1000 mM, and 2000 mM) or by 0.5 M acetic acid, [pH 3.4]. Eluted proteins were precipitated with TCA. Proteins were solubilized in SDS-PAGE

sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting. For making whole-cell lysates, cells derived from 2 ml of culture were washed with H_2O and sonicated for 20–30 s in 35 μl of SDS-PAGE sample buffer. Samples were then incubated at 75°C for 15 min and proteins separated by SDS-PAGE.

Nitrocellulose membranes were blocked with 5% skim milk powder in PBS containing 0.1% Tween-20. Specific rabbit polyclonal antibodies were used to detect Nup53p, Kap121p (Marelli et al., 1998), Nup49p, Nup57p (Grandi et al., 1995; kindly provided by E.C. Hurt, University of Heidelberg, Germany), GFP (provided by M. Rout, The Rockefeller University), Clb2p (Santa Cruz Biotechnology), and Gsp1p. Pds1-HA was detected using monoclonal 12CA5 antibodies. Either HRP-conjugated, donkey antirabbit, or sheep antimouse secondary antibodies and the ECL system (Amersham Biosciences) were used to detect primary antibodies and protein A moieties.

In Vitro Binding Assays

An *E. coli* BL21 strain transformed with either pGEX-KAP121, pGPHO4, pGNP53, or pG1-375 was grown to mid-log phase and induced with 1 mM IPTG for 3 hr to synthesize GST fusions of Kap121p, Pho4p, Nup53p, and a C-terminal truncation of Nup53p, 1-375. All GST-fusions were purified on glutathione Sepharose (GT) beads according to the manufacturer's instructions (Amersham Biosciences) using 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Tween-20, and 50 mM Tris, [pH 7.5] as a lysis and wash buffer. Kap121p was purified as described previously (Marelli et al., 1998) and incubated with GT-beads preloaded with GST-Pho4p for 1 hr at 4°C. After washing, this complex was released from the GT using the PreScission Protease (Amersham Biosciences), which recognizes a site between GST and Pho4p. The Kap121p/Pho4p complex (~10 μg) was then incubated for 1 hr at 4°C with 10 μl of GT beads preloaded with ~5 μg of GST-Nup53p, or GST-1-375. The beads were washed and eluted with SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and detected with Bio-Safe Coomassie (BioRad).

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